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Effect of Penicillin on the Multiplication of Meningopneumonitis Organisms (*Chlamydia psittaci*)

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Although formation of infectious particles of meningopneumonitis organism in L cells was completely inhibited by 1 or more units of penicillin per ml, multiplication of reticulate bodies was observed, by light microscopy, in the presence of 200 units of penicillin per ml in stained smears of infected cells. When reticulate bodies were purified from cultures containing penicillin after 18, 30, and 45 hr of incubation, continuously increasing yields were obtained. When penicillin was added to infected cultures 0 to 15 hr after infection, no increase in infectivity was observed at 40 hr, but when antibiotic was added between 20 and 35 hr, partial synthesis of infectious particles was observed at 40 hr. On the other hand, removal of penicillin from an infected culture before 15 hr after infection did not affect the final yields of infectivity when assayed at 40 hr, but elimination of penicillin after 20 hr resulted in a decrease in infectivity. In suspensions of ^{32}P-labeled purified reticulate bodies grown in cultures containing penicillin and harvested 18 and 40 hr after infection, the ^{32}P distributions obtained by acid fractionation were similar to those of reticulate bodies from penicillin-free cultures. Cell membranes of reticulate bodies were also prepared from 40-hr cultures with penicillin. The size and shape of purified membranes, as seen by electron microscopy, and their amino acid compositions were similar to membranes prepared from reticulate bodies grown without penicillin, except that very small structures were observed in membranes from cultures containing penicillin. These results indicated that penicillin does not inhibit reproduction of reticulate bodies and formation of their cell membranes, but does inhibit the formation of elementary body cell envelopes.

The inhibition of growth of organisms of the psittacosis group (*Chlamydia*) by penicillin has been amply confirmed but its mechanism and site of action have not been demonstrated. These organisms undergo a definite developmental cycle, originally described by Bedson from observation with light microscopy (1) and recently confirmed in fine detail by Higashi and others (2, 3). The resistant extracellular infectious form of the organism (elementary body, EB) is converted, after cell penetration, into a larger, fragile developmental reticulate form (reticulate body, RB) which multiplies by fission, with the new RB finally undergoing a process of maturation to form EB. In 1950, Weiss (13) reported that, when penicillin was injected into eggs that were already heavily infected with feline or murine pneumonitis organisms, evidence was obtained by light microscopic examination that division of the organisms was inhibited; however, an abnormal growth, without apparent division, of some form of the organism, presumably the developmental reticulate form, was observed.

Tamura and his colleagues have recently developed methods by which highly purified suspensions of each form of the organism, EB and RB, can be prepared in quantity, and comparative studies have been done on the morphology and composition of each (8-11). This ability to separate the two forms during stages of development suggested to us a study of the effect of penicillin on the organism in each phase. This is of particular interest because muramic acid has been reported to be a constituent of the psittacosis organism by Jenkin (4) and by Perkins and

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Allison (7), whereas we have been unable to detect the amino sugar in purified cell envelopes of the meningopneumonitis (MP) organism. If this substance does occur, it comprises less than 0.2% of the cell wall and is not a major component, although the MP organism is sensitive to penicillin (6).

In this paper, we report a series of experiments in which the sensitivity of the MP organism to penicillin, the effect of penicillin on the yield of both EB and RB, and the effect of penicillin on cell envelope morphology have been studied.

MATERIALS AND METHODS

Organism and cells. MP strain of *Chlamydia psittaci* was cultured in suspended L cells. The methods for propagation were essentially the same as those described previously (8). For these experiments, stock inoculum was prepared by inoculating 5 ml of infected culture supernatant fluid into a 60-ml suspension of L cells containing 10^6 cells per ml. This suspension was diluted to 120 ml with fresh medium, divided into two flasks of 60 ml each, then incubated at 37 C on a rotary shaker for 48 to 72 hr. Cells were examined in stained smears; when most of the cells showed massive infection, the cultures were stored at 4 C. Immediately before use as inoculum, the organisms were concentrated and partially purified by differential centrifugation, and the MP pellets were resuspended in fresh medium.

For inoculation, L cells in suspension were collected by centrifugation, washed once with phosphate-buffered saline (PBS), and resuspended in the MP inoculum at a final concentration of 2×10^7 cells per ml. After 1 hr of adsorption at 37 C, the infected cells were washed three times with PBS and once with fresh medium. The cells were then diluted to contain 6×10^5 cells per ml in fresh medium and were incubated at 37 C on a rotary shaker. The media used for L cell cultivation and infected cell maintenance were the same as those used by Tamura and Higashi (8).

Titration of infectivity. A slight modification of the method of Chappell and Manire was used (Bacteriol. Proc., p. 118, 1965). L-cell monolayers were grown in 60-ml plastic petri dishes in a medium consisting of 0.15% yeastolate, 0.001% phenol red, 0.2% streptomycin, 0.01% kanamycin, and 16.5% horse serum in Hanks balanced salt solution (BSS), and were incubated in a 5% CO_2 atmosphere at 37 C.

The monolayers were washed once with PBS and were inoculated with 0.1 ml of MP suspension. After adsorption for 4 hr at 37 C with shaking every 20 min, 4 ml of agar overlay was added. After incubating the plates for 7 days at 37 C in 5% CO_2, the second overlay was added; incubation continued until the 14th day, when plaques were counted. The first overlay medium contained 1% agar, 0.15% yeastolate, 0.02% streptomycin, 0.01% kanamycin, and 16.5% horse serum in medium 199 made up in Hanks BSS. The second overlay medium was the same, with 0.006% neutral red added.

Other methods. The methods for preparation of electron micrographs have been reported previously (5). All specimens were shadowed with platinum-palladium alloy and were examined in an Akashi TRC 50 microscope.

The methods used for labeling organisms with ^{32}P for acid fractionation, for amino acid analysis, and for dry weight determination have been described previously (6).

RESULTS

Penicillin sensitivity of organism. L-cell suspension cultures were infected with MP at a multiplicity of 10 plaque-forming units (PFU)/cell. After adsorption, residual inoculum was removed and the cells were washed by repeated centrifugation. Equal amounts of infected cells were incubated in separate flasks in medium containing penicillin at concentrations of 0 to 50 units per ml. After 40 hr, 10 ml of each culture was homogenized for 10 min in an ice bath with a Teflon grinder, then centrifuged at 1,000 rev/min for 10 min to remove cell debris. The yields of infectious organisms were assayed by plaque count (Table 1). About 50% inhibition was observed in medium containing 0.1 unit of drug per ml, and at least 98% inhibition occurred when 1 or more units of drug was added. Infectious organisms in the suspension containing high concentrations of penicillin resulted from the residual inoculum which could not be removed by washing but which did not penetrate cells and convert to RB. It can be assumed that 100% inhibition occurred with 1 unit of drug per ml and that the addition of 50 units per ml gave no further reduction.

Microscopic observations. A suspension of L cells was infected with MP, then divided into two flasks; one flask contained 200 units of penicillin per ml and the other did not contain drug. At various intervals, cells from each suspension were carefully stained with Macchiavello stain and were observed by light microscopy. In control cultures, blue-stained developmental RB were seen at 8 to 10 hr after infection. Between 10 and 25 hr, these bodies increased rapidly in number and size; by 25 hr, red-stained EB appeared within the clumps. After 30 hr, a rapid increase occurred in the number of red-staining EB, which filled the cytoplasm by 35 hr.

In cultures containing penicillin, similar developments were observed up to 20 hr, although the clumps of RB were somewhat smaller. Between 20 to 40 hr, the number of blue-staining clumps of RB continued to increase, but formation of small, red-staining clumps of EB was never observed. At 40 to 46 hr, the cytoplasm of the cells was filled with RB and cell lysis was seen. This finding strongly suggested that regular reproduction occurs in penicillin, but maturation into the infectious dense form is inhibited.

TABLE 1. *Effect of penicillin on the yield of infectious MP organisms during a 40-hr growth period*

Concn of penicillin	PFU/ml at 40 hr after infection	Per cent of control
units/ml		
None	149×10^5	100.0
0.05	112×10^5	75.2
0.1	79×10^5	53.0
0.5	19.4×10^5	13.0
1.0	1.75×10^5	1.2
5.0	2.35×10^5	1.6
50.0	1.76×10^5	1.2

Effect of penicillin on growth rate of developmental forms (RB). To determine the rate of growth of MP in the presence of penicillin, RB were recovered and purified from infected cultures containing penicillin at different times after infection and the yield was measured by dry weight determination.

A 600-ml suspension culture of L cells was infected with MP in the usual way and was equally divided into four spinner culture bottles. One bottle was incubated without penicillin, and to each of the other three bottles we added 200 units of penicillin per ml. At 18 hr after infection, before any formation of EB occurred, the control and one penicillin culture were harvested. The remaining cultures were harvested at 30 and 45 hr after infection. RB were recovered and purified from each culture by methods previously reported (10). The purified RB from the sharp band in the potassium tartrate gradient were diluted threefold in water and were centrifuged at 10,000 rev/min for 1 hr. The pellets were washed three additional times in distilled water, and the final pellets were dried in stainless-steel dishes. The dry weight yields of RB (Table 2) give definite evidence of continued multiplication of the organism for more than 30 hr in penicillin cultures.

To study the kinetics of inhibition by penicillin, two experiments were done. In the first, 450 ml of infected cells was incubated at 37 C in a spinner flask. At intervals, 50 ml of cell suspension was transferred to a 250-ml flask and penicillin was added to a concentration of 200 units per ml. Each suspension was then incubated on a shaker until 40 hr after infection, at which time the infectivity of the suspension was assayed as above. The results are presented in Fig. 1; the PFU per ml at 40 hr are plotted against the time of penicillin addition. When penicillin was added before 20 hr, complete inhibition occurred, but, when the drug was added after 20 hr, partial or no inhibition was observed.

TABLE 2. *Effect of penicillin on the production of RB of MP organisms*

Culture	Penicillin concn (units/ml)	Time of harvest (hr after infection)	Total RB yield (mg, dry wt)
A	0	18	4.2
B	200	18	3.5
C	200	30	7.4
D	200	45	13.3

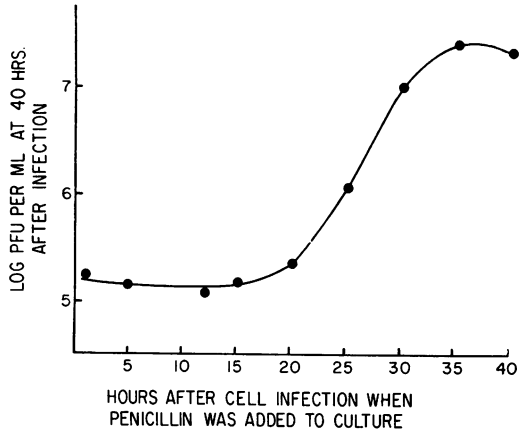

FIG. 1. *Effect of penicillin on the yield of infectious MP organisms at 40 hr after cell infection. The drug was added to infected cultures at the intervals indicated by points on the graph. All cultures were harvested and titrated after a total of 40 hr of incubation.*

In a second experiment, 450 ml of MP-infected cells was incubated in a medium containing 200 units of penicillin per ml. At intervals, 50 ml of the cell suspension was removed, and the cells were washed twice with PBS by centrifugation at 1,000 rev/min. The cells were resuspended in 50 ml of medium without penicillin and were incubated in a 250-ml flask on a rotary shaker at 37 C. At 40 hr after infection, the infectivity of each culture was measured. The results are shown in Fig. 2; the PFU per ml at 40 hr are plotted against the time of removal of penicillin. When the drug was removed from cultures before 20 hr after infection, no decrease in yield was observed, but, when penicillin was removed 25 hr or more after infection, the formation of infectious particles was clearly inhibited. It has been amply demonstrated that, when MP organisms are cultivated in L cells, maturation to form infectious EB begins at about 18 to 20 hr after infection (11). In both experiments described above, the effect of penicillin was observed primarily in the 15- to

20-hr period, which would indicate that the drug inhibits this maturation.

Chemical composition and morphology of RB and their membranes cultured in penicillin. To determine whether RB harvested from cells grown in the presence of penicillin differ from those grown without the drug, L cells infected with MP were suspended in medium containing ^{32}P (final concentration, 1 μc per ml), and the cell suspension was divided into three flasks. Penicillin (200 units per ml) was added to two of the cultures, and all three were incubated on a rotary shaker at 37 C. RB were harvested and purified from an 18-hr culture without penicillin and from 18-hr and 40-hr cultures with penicillin. Each preparation was subjected to acid fractionation, and the ^{32}P content in cold acid-soluble, lipid, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and residual fractions was determined. ^{32}P distribution was similar in the three preparations (Table 3). In purified RB, the ^{32}P content of RNA is always higher than the ^{32}P content of DNA (11); we found that the ratio of RNA to DNA was 3:1 in both 18-hr cultures and 2:1 in the 40-hr culture. The total nucleic acid ^{32}P was slightly lower in the 18-hr culture with penicillin but much higher in the 40-hr culture than in the 18-hr penicillin-free culture, indicating continuous synthesis of nucleic acid in the presence of the drug.

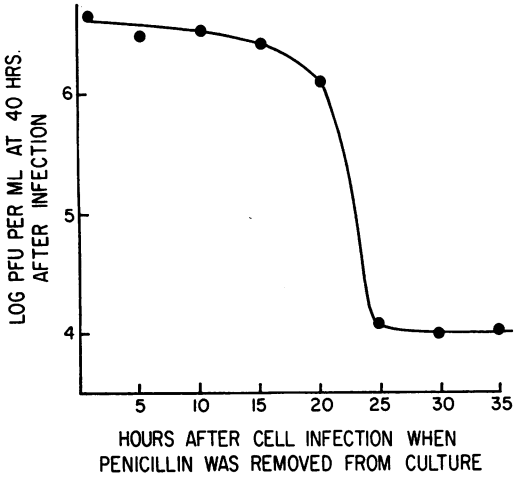

FIG. 2. *Effect of penicillin on the yield of infectious MP organisms at 40 hr after cell infection. The drug was removed from infected cultures at the intervals indicated by points on the graph. All cultures were harvested and titrated after a total of 40 hr of incubation.*

Membranes were prepared from RB grown in penicillin medium for 40 hr by methods previously reported (10); such purified membranes are shown in Fig. 3. These membranes are very flat and quite similar to those prepared from the 18-hr culture without drug. One striking feature, however, is the presence of a relatively large number of small membrane-like structures both inside and outside the RB membranes. The significance of these structures is not known.

The amino acid content of purified membranes prepared from RB grown in penicillin for 40 hr was determined. In Table 4, the results are compared with the composition of dense body cell envelopes and RB membranes from 18-hr cultures without penicillin, as reported previously (6, 10). The cell envelopes of EB contained all of the common amino acids. The cell membranes of 18-hr RB were similar but had no methionine or cystine, whereas the 40-hr RB membranes contained a very small amount of methionine.

DISCUSSION

This reexamination of the effects of penicillin on psittacosis organisms is of special interest. The developmental cycle of the organism has been elucidated by thin-section electron microscopy (2). Tamura and his associates (8, 11) have developed methods for the preparation of purified suspensions of both the infectious dense EB and the intermediate RB, which is the reproductive form of the organism. From light microscopy studies of infected yolk sac tissue, Weiss (13) has concluded that the intermediate form develops

TABLE 3. *Effect of penicillin on ^{32}P distribution in RB of MP organisms*

Fractions	RB from 18-hr cultures without penicillin		RB from 18-hr cultures with penicillin		RB from 40-hr cultures with penicillin	
	Total counts/min	Per cent	Total counts/min	Per cent	Total counts/min	Per cent
Acid-soluble	3.08×10^4	8.9	2.48×10^4	9.5	13.0×10^4	13.2
Lipid	12.1×10^4	34.7	10.6×10^4	40.7	44.6×10^4	45.1
RNA	14.5×10^4	41.7	9.62×10^4	36.8	25.6×10^4	25.9
DNA	4.51×10^4	13.0	3.17×10^4	12.1	13.3×10^4	13.4
Residual	0.59×10^4	1.7	0.26×10^4	1.0	2.37×10^4	2.4

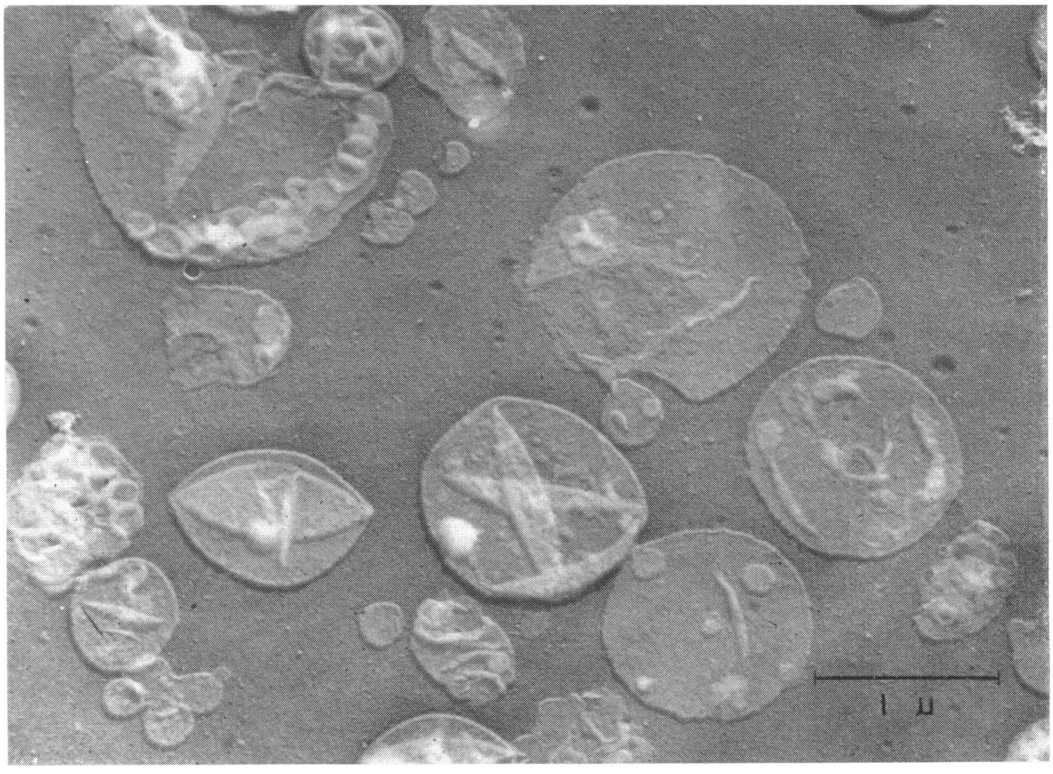

FIG. 3. *Cell envelopes of RB prepared from MP organisms grown for 40 hr in medium containing 200 units of penicillin per ml.*

and increases in size but does not divide. More recently, Walz (12) reported similar observations. However, neither of these studies included the isolation and morphological study of individual organisms grown in penicillin, and it is not possible to discern the structure of the developing forms with stained preparations in light microscopy.

When L cells infected with MP in the presence of penicillin were stained with Giemsa, we were able to observe enlarged "plaques" with light microscopy; smaller darkly stained areas could usually be seen within these bodies. In electron micrographs of purified RB and their membranes prepared from cultures containing penicillin, such enlarged bodies were not seen, perhaps because they are not intact membranous bodies. Higashi (2) has reported the presence of large RB containing two or three daughter particles in cells studied by thin-section electron microscopy. However, the above evidence seems to indicate that these organisms multiply in essentially the same way whether penicillin is present or absent.

MP organisms in L cells undergo rapid growth and cell division without any formation of in-

TABLE 4. *Amino acid composition of membranes of EB and RB*[a]

Amino acid	Membranes of EB	Membranes of RB from 18-hr cultures without penicillin	Membranes of RB from 40-hr cultures with penicillin
Alanine	9.9	11.0	10.7
Arginine	3.7	3.0	3.0
Aspartic acid	12.2	12.1	12.0
Half cystine	4.2	0	0
Glutamic acid	8.7	8.9	8.0
Glycine	9.1	9.6	9.0
Histidine	1.3	1.2	1.2
Isoleucine	3.6	5.4	5.0
Leucine	6.4	7.9	8.0
Lysine	5.4	4.8	5.2
Methionine	0.9	0	0.4
Phenylalanine	3.8	4.2	4.1
Proline	6.6	6.4	6.0
Serine	9.4	9.7	10.1
Threonine	7.6	8.0	9.2
Tyrosine	3.1	2.7	3.1
Valine	4.2	5.3	5.0

[a] All results are shown as micromoles per cent of total amino acids.

fectious dense forms until 18 to 20 hr after infection; at this time such organisms begin to appear in increasing numbers. This finding has been verified by both microscopic observations, as reported in this paper, and by the isolation of the organisms at intervals after infection. The observation that penicillin has little effect on the growth of the organism up to 18 hr but completely inhibits formation of the infectious form of the organism indicates that penicillin prevents maturation but does not prevent further reproduction of the organism. That this is in fact the case is indicated by the observation that, although penicillin does cause a slight reduction in the dry weight yield of reticulate bodies at 18 hr, there is a significant increase in numbers at 30 hr and the final yield at 40 hr in penicillin is three to four times greater than the 18-hr yield without penicillin. Similar evidence was obtained with ^{32}P-labeled RNA and DNA from RB grown in penicillin. Synthesis of nucleic acid continued throughout the 40-hr growth period.

We have recently described certain physical and chemical properties of the envelopes of both EB and RB of MP (6, 10). The infectious dense form has a relatively heavy, rigid, strong cell wall that contains most of the naturally occurring amino acids, including methionine and cystine, and significant amounts of phospholipid. In many ways the envelopes resemble bacterial cell walls, although we have not been able to detect muramic acid in these preparations. If muramic acid is present, our evidence suggests that it comprises $<0.2\%$ of the dry weight.

In contrast, the envelope surrounding the developmental reticulate form is fragile, thin, nonrigid, and easily broken. These membranes contain no methionine or cystine and have little phospholipid.

RB envelopes prepared from 18-hr cultures of MP-infected L cells with or without penicillin and from 40-hr cultures with penicillin do not differ markedly. The only difference detected in these studies was the appearance, at 40 hr, of a very small amount of methionine; in addition, in electron micrographs, small internal membrane structures, the nature of which is unknown, were observed.

We can thus conclude that penicillin prevents the maturation of the organism by inhibiting EB envelope synthesis. It is unlikely that the outer envelope or cell wall is removed from the EB after cell infection to form RB, but rather that the outer membrane loses some constituents, which are replaced at maturation, or changes in its molecular configuration. We are now in the process of isolating and identifying the amino sugars and other components of these membranes; hopefully, this will help elucidate the structure of these envelopes and the mechanism of action of penicillin in this system.

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